HIV Gag mRNA Transfection of Dendritic Cells (DC) Delivers Encoded Antigen to MHC Class I and II Molecules, Causes DC Maturation, and Induces a Potent Human In Vitro Primary Immune Response[1]

Drew Weissman,[2]* Houping Ni,* David Scales,* Annie Dude,* John Capodici,* Karen McGibney,* Asha Abdool,* Stuart N. Isaacs,* Georgetta Cannon,* and Katalin Karikó†

Dendritic cells (DC) are the major APCs involved in naive T cell activation making them prime targets of vaccine research. We observed that mRNA was efficiently transfected, resulting in superior translation in DC compared with other professional APCs. A single stimulation of T cells by HIV gag-encoded mRNA-transfected DC in vitro resulted in primary $CD4^+$ and $CD8^+$ T cell immune responses at frequencies of Ag-specific cells (5–12.5%) similar to primary immune responses observed in vivo in murine models. Additionally, mRNA transfection also delivered a maturation signal to DC. Our results demonstrated that mRNA-mediated delivery of encoded Ag to DC induced potent primary T cell responses in vitro. mRNA transfection of DC, which mediated efficient delivery of antigenic peptides to MHC class I and II molecules, as well as delivering a maturation signal to DC, has the potential to be a potent and effective anti-HIV T cell-activating vaccine. *The Journal of Immunology,* **2000, 165: 4710–4717.**

Vaccines that induce potent T cell immunity are under active investigation for diseases such as HIV, cancer, and tuberculosis (1). Dendritic cells (DC)[3] are the most potent APCs in the immune system and are critical in the initial recruitment and activation of naive T cells (2–4). As DC are the principal, if not only, APC involved in primary T cell immune responses, the first step of vaccination involves the delivery of candidate Ag to DC. The immune responses necessary for protection from HIV infection are unknown, but analyses of long term nonprogressors, HIV-infected individuals with normal and stable CD4 counts, demonstrate strong $CD4^+$ and $CD8^+$ T cell immunity against HIV Ags (reviewed in Refs. 5–7). Thus, it is theorized that vaccines capable of inducing broad T cell immunity against HIV will delay HIV progression in infected individuals or alter disease course in those who become infected after vaccination. HIV vaccines currently in clinical trials (reviewed in Refs. 8 and 9) rely on the delivery of Ag to DC after immunization by direct local uptake of delivered protein or infection or transfection of local nonlymphoid cells with resultant delivery of Ag to DC, and possibly direct DC transfection (10–13). In vitro expansion of DC followed by HIV protein or peptide pulsing, maturation, and reinfusion has also been described (14). Unfortunately, at present, immunity induced by these HIV vaccine approaches has demonstrated at best moderate induction of CTL and CD4 helper activity.

Multiple in vivo and in vitro studies have used DC-based vaccines with various forms of Ag delivery to induce immunity, and, in some cases, potent antitumor responses have been observed (reviewed in Ref. 15). The form in which Ag is delivered to APC influences the efficiency and pathway of Ag presentation used and the subsequent quality of T cell activation. Current methods of direct delivery of Ags to DC in vitro include peptide or protein loading, DNA transfection (reviewed in Refs. 16–18), and viral vector infection (19–22). Potential confounding factors of these methods of delivery of Ag to DC include the need to tailor peptides to the MHC haplotype (reviewed in Ref. 23); the low efficiency of DNA transfection of primary cells and the potential development of stable transfectants (24–26); the limitation of added whole protein to primarily load-only MHC class II molecules, and, especially with HIV proteins, a direct effect on immune cell function (reviewed in Refs. 27 and 28); and viral vector effects on DC function (29, 30). Most of these pitfalls can be avoided by Ag delivery to DC in an mRNA-encoded form. mRNA transfection for Ag delivery to DC has been used to induce potent T cell-based antitumor immunity in vivo and in vitro (15, 31–34).

In this report, we demonstrate that transfecting DC with mRNA encoding the HIV core protein gag results in potent primary $CD4^+$ and $CD8^+$ T cell immune responses in an in vitro system with the generation of frequencies of Ag-specific cells similar to that observed in in vivo model systems (35). We demonstrate that this is due to extremely efficient delivery of encoded Ag to DC with loading of antigenic peptides onto MHC class I and II molecules and the induction of a DC maturation signal by mRNA transfection.

Materials and Methods

Materials

PBMC were obtained by leukopheresis of HIV-uninfected volunteers through an Institutional Review Board-approved protocol. PBMC were purified by Ficoll-Hypaque density gradient purification. Cells were either used immediately or cryopreserved in RPMI 1640 (Life Technologies, Gaithersburg, MD) medium with 10% NHS (Sigma, St. Louis, MO) and

Division of *Infectious Diseases and †Neurosurgery, University of Pennsylvania, Philadelphia, PA 19096

Received for publication May 2, 2000. Accepted for publication July 19, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants R01-HL-62060-1 and R21-AI-45318-01A1.

[2] Address correspondence and reprint requests to Dr. Drew Weissman, Division of Infectious Diseases, University of Pennsylvania, 536 Johnson Pavilion, Philadelphia, PA 19104. E-mail address: dreww@mail.med.upenn.edu

[3] Abbreviations used in this paper: DC, dendritic cell(s); NHS, normal human serum; β-gal, β-galactosidase.

0022-1767/00/$02.00

10% DMSO (Sigma). CD4-peridinin chlorophyll protein, CD69-FITC, anti-human IFN-γ-PE, HLA-DR-FITC, CD14-FITC, CD11c-FITC, matched isotype controls (Becton Dickinson Immunocytometry Systems, San Jose, CA), CD80-FITC (Research Diagnostics, Flanders, NJ), CD83-PE (Caltag, Burlingame, CA), CD86-CyChrome (PharMingen/Becton Dickinson, San Jose, CA), IL-4, IL-7, GM-CSF, and TNF-α (R & D Systems, Minneapolis, MN), IL-2 (obtained from the National Institute of Allergy and Infectious Diseases AIDS Reference and Reagent Program), and PGE_3 (Cayman Chemical, Ann Arbor, MI) were used.

DC preparation

The purification of DC from PBMC used the method originally described by Sallusto and Lanzavecchia with minor modification (36, 37). Briefly, monocytes were purified from PBMC by discontinuous Percoll gradient centrifugation. The low density fraction (monocyte enriched) was depleted of B, T, and, in certain experiments, NK cells using magnetic beads (Dynal, Lake Success, NY) specific for CD2, CD16, CD19, and CD56. This resulted in highly purified monocytes as determined by flow cytometry using anti-CD14 (>95%) or anti-CD11c (>98%) mAb. To generate immature DC, purified monocytes were cultured in either RPMI 1640 supplemented with glutamine (2 mM), HEPES (15 mM), and 1% NHS (Sigma) or in AIM V serum-free medium (Life Technologies), supplemented with GM-CSF (50 ng/ml) and IL-4 (100 ng/ml). TNF-α (1 ng/ml) and PGE_3 (500 nM) were used for DC maturation when indicated.

Plasmid constructs

pCMV-lacZ (β-galactosidase (gal)) (Clontech, Palo Alto, CA), obtained from Benjamin Doranz (University of Pennsylvania, Philadelphia, PA), was used for plasmid transfection using the same method described below for mRNA. The following plasmids were used as templates for in vitro mRNA transcription: pSFV3-lacZ was purchased from Life Technologies, pT7-TEV (the leader sequence of the tobacco etch viral genomic RNA)-luciferase-A_{50} and pT7-luciferase-A_{50} were obtained from Dr Daniel Gallie (University of California, Riverside, CA), pDAB72 was obtained from the National Institute of Allergy and Infectious Diseases AIDS Reference and Reagent Program (38), and pCCR5 was a gift from Dr. Ben-Hur Lee (University of Pennsylvania). To construct pT7-TEV-gag-A_{50} the *Nco*I-*Bam*HI fragment containing the 1539-bp HIV gag coding region was isolated from pDAB72. This fragment was cloned into pT7-TEV-luciferase-A_{50} in place of the *Nco*I-*Bam*HI luciferase insert. Plasmids were purified using Quantum Prep (Bio-Rad, Hercules, CA), quantitated by OD at 260 nm, and sequenced.

In vitro transcription

mRNA transcription was performed on a linearized plasmid template using Message Machine kits (Ambion, Austin, TX) according to the manufacturer to generate m^7GpppG-capped mRNAs. Plasmid pSFV3-lacZ was linearized with *Spe*I, and SP6 RNA polymerase was used to synthesize capSFV-lacZ-A_{90} (SFV3), an 11,344-nt-long self-replicative mRNA. This transcript encodes the nonstructural polyprotein of the Semliki Forest virus and the bacterial β-gal gene of *Escherichia coli*, and has a 90-nt poly(A) tail (39). The plasmid pT7-TEV-luciferase-A_{50} was linearized with *Nde*I, and T7 RNA polymerase was used to transcribe cap-TEV-luciferase-A_{50} (TEV-luciferase) (40). A structurally similar transcript, cap-TEV-gag-A_{50} (TEV-gag) was obtained from the related *Nde*I-linearized pT7-TEV-gag-A_{50} plasmid. This transcript encodes HIV gag p55 obtained from BH10 (41). Another gag-encoding mRNA was generated from plasmid pDAB72 after linearizing with *Bam*HI and transcribing with T7 RNA polymerase. To synthesize cap-gag-A_n (gag), an ~200-nt-long poly(A) tail was added using yeast poly(A) polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer. mRNA used as controls included cap-luciferase-A_{50}, cap-anti-sense CCR5-A_n, and poly(A) homopolymer (Sigma).

Purification of the transcripts was performed by DNase I digestion followed by LiCl precipitation and 75% ethanol wash. At least three different batches of mRNA were generated and used. The quality of each batch of mRNAs was tested by agarose gel electrophoresis for degradation and the presence of contaminating DNA template. RNA samples were quantitated by measuring OD at 260 nm. Samples were stored in siliconized tubes at −20°C at 0.5–1.0 μg/μl final concentrations.

The characterized mRNA or plasmid was complexed to Lipofectin (Life Technologies). To enhance the efficiency of mRNA transfection and to decrease lipid-related cytotoxicity, the complexing was performed in the presence of phosphate buffer as described (42). Lipofectin (0.2 μg) was preincubated in 40 mM phosphate buffer and 0.1 μg/ml BSA before complexing to 0.2 μg of mRNA in a final volume of 50 μl. Aliquots (50 μl) of the mRNA/lipid complexes were added directly to serum-free medium-washed DC, B cells, monocytes, 293T cells, or $CD4^+$ T cells (10^5 cells) for 1 h, and were then resuspended in fresh medium. Aliquots of mRNA complexed with Lipofectin used for transfecting DC were found not to contain endotoxin using the *Limulus* amebocyte lysate gel clot assay (<3 pg/ml) (University of Pennsylvania, Department of Genetics, Cell Center Service Facility).

Recombinant vaccinia gag

A fragment of gag from the HXB2 clone of prototype HIV strain IIIB (amino acid 29–498) was generated by PCR using primers that introduced an appropriate initiating ATG and termination codon and allowed cloning into the *Bgl*II/*Sma*I sites of pSC65 (43). The resulting plasmid allowed for recombination into the vaccinia virus thymidine kinase gene (TK) and the expression of the cloned gag to be driven by a vaccinia virus strong synthetic early/late promoter. Recombinant vaccinia virus-encoding gag was isolated and plaque-purified following standard techniques on a TK^- cell line (143B, American Type Culture Collection, Manassas, VA) in the presence of bromodeoxyuridine and X-gal (44). Western blots of infected cell lysates showed that gag was produced and recognized by a rabbit polyclonal antiserum (AIDS Reference and Reagent Program) (45). This virus was used to infect DC in suspension at a multiplicity of infection of ~2 PFU/cell for 2 h. Cells were then washed in medium and cultured overnight.

Cell cultures

Immature DC were pulsed with mRNA or protein for 1 h in serum-free medium; in certain cases, DC were matured 6 h later with TNF-α and PGE_3. Peptide-pulsed DC were prepared by first maturing and then pulsing DC with a series of 49 different 20-mer overlapping polypeptides corresponding to the p55 protein of HIV-gag (each peptide at 1 μg/ml) (obtained from the National Institute of Allergy and Infectious Diseases AIDS Reference and Reagent Program) for 1 h at 37°C followed by washing. Autologous T cells and $CD8^+$ T cells were purified by negative selection using cell separation columns following the manufacturer's instructions (R&D Systems). DC, 24 h after pulsing, were cultured with autologous T cells at a ratio of one DC per 10 T cells. In certain experiments, IL-2 (20–40 U/ml) and IL-7 (5 ng/ml) were added.

B cells were purified by positive selection using CD19 dynabeads (Dynal) and stimulated with pokeweed mitogen (2.5 μg/ml; Sigma) for 1–3 days before luciferase mRNA transfection. $CD4^+$ T cells were stimulated with PHA (4 μg/ml; Sigma) for 3 days before luciferase mRNA transfection. 293T cells were obtained from Dr. Ben-Hur Lee (University of Pennsylvania) and grown in DMEM (Life Technologies) supplemented with glutamine and 10% FCS (HyClone, Ogden, UT).

Reporter gene product analyses

Luciferase enzymatic activity was measured by lysing cells in cell culture lysis reagent (Promega, Madison, WI), adding luciferase substrate (Promega), and measuring in a luminometer (MLX; Dynex, Chantilly, VA or LB9506; Wallac, Gaithersburg, MD) as described by the manufacturer. A standard curve of luciferase protein (Promega) was used to calculate luciferase protein concentration from relative light units. Immunohistochemistry for bacterial β-gal was performed on cytospun (model 7620; Wescor, Logan, UT) cells fixed with 50% acetone/50% MeOH for 30 min at −20°C with a specific mAb (KM-90; Accurate Chemicals and Scientific, Westbury, NY) and the Dako Catalyzed Amplification System (Dako, Carpinteria, CA). p24 (gag) protein was quantitated by ELISA (Beckman Coulter, Fullerton, CA). Supernatants were directly assayed. Cell lysates for p24 analysis were prepared by lysing PBS-washed cells in 50 μl luciferase lysis buffer and adding 150 μl RPMI 1640 with 10% FCS before analysis. β-gal activity in lysed cells was measured with Galacto-Star (Tropix/PE Biosystems, Foster City, CA).

Effector cell generation for intracellular cytokine analysis and CTL activity

Fourteen days after primary stimulation, T cells were restimulated with DC loaded with Ag similar to the primary stimulation at ratios ranging from 10–50 T cells per DC in 1.5-ml Eppendorf tubes. Brefeldin A (10 μM; Sigma) was added 1 h later, and cells were washed with PBS and cultured in PBS 0.05% EDTA for 15 min after 6 h from the start of the culture. Six-hour activated T cells were then stained with CD4-peridinin chlorophyll protein, fixed in 2% paraformaldehyde (Sigma) for 15 min, washed, permeabilized with 0.25% saponin (Sigma) for 15 min, stained with CD69-Fl and anti-IFN-γ-PE, and fixed with FACS lysing buffer (Becton Dickinson). Propidium iodide (50 μg/ml) was added to certain experiments

to analyze cell death. Stained cells were analyzed on a FACScan (Becton Dickinson); 50,000 events were analyzed per sample.

Vaccinia-gag secondary stimulations were performed by similar procedures as described above. Analysis of an immune response in vaccinia-gag stimulated T cells was performed with gag mRNA-loaded DC to distinguish vaccinia-specific responses.

For CTL assays (14, 34, 46), $CD8^+$ T cells were obtained by negative selection 5 days after effector cell stimulation. Targets for CTL activity were DC transfected with control or HIV gag-encoding mRNA (34) or pulsed with HIV peptides and loaded with 25 μM calcein AM (Molecular Probes, Eugene, OR), a fluorescent dye that is released when membrane integrity is compromised, for 45 min. DC targets were cocultured with $CD8^+$ T cells at a range of E:T ratios (50:1–12.5:1). Cytotoxicity was measured by analyzing the supernatant for calcein in a fluorometer (MFX; Dynex) (485 nm excitation, 535 nm emission) after 3 h of culture. Controls including 100% lysis (maximum release) and target cells alone (spontaneous lysis) were included, and cytotoxicity was expressed by the following equation: percent specific lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release).

Analysis of mRNA-induced DC maturation

Immature DC were transfected with gag mRNA complexed with lipofectin or treated with lipofectin without nucleic acid. Twenty hours later, cells were stained with CD80-FITC, CD86-CyChrome, and CD83-PE and analyzed on a FACScan.

Results

Transfection of DC with mRNA-encoding reporter proteins

Ag presentation and T cell activation, in addition to being affected by the route of Ag delivery (MHC class I vs II pathways), is dependent on the amount of Ag delivered and the number of DC presenting the Ag (10, 11, 13, 47–49). We first determined the kinetics of protein production after luciferase-encoding mRNA transfection of DC. Immature DC were transfected with increasing concentrations of lipofectin-complexed TEV-luciferase mRNA and assayed 4 h later. This time point was chosen because we found that after transfection, luciferase activity reached a maximum at 3–8 h, and significantly decreased by 24 h (Fig. 1*A*). High levels of luciferase protein, up to 508 pg/10^5 DC, were produced with 0.2 μg/50 μl of mRNA (Fig. 1*B*). In a comparison of DC to other professional APC and $CD4^+$ T cells, DC produced over 1000 times more protein when transfected with the same amount of luciferase mRNA (Fig. 1*C*). As luciferase protein denatures quickly after translation and loses enzymatic activity (50), the measure of enzymatic activity corresponds to active translation of encoded mRNA, suggesting that mRNA transfection of DC results in transient, high level protein production.

It has been established that the efficiency of T cell activation by DNA-mediated Ag delivery to DC correlates with the number of APC actively producing encoded protein (10, 11, 13) and is typically low (24–26). To determine the transfection efficiency of DC by mRNA, we used a bicistronic mRNA (SFV3) encoding the SFV nonstructural proteins required for RNA replication, a subgenomic promoter, and the reporter β-gal. Upon introduction into the cytoplasm, the translated polyprotein from the first cistron replicates both cistrons, producing high levels of β-gal protein. This system was used because experiments using β-gal- or luciferase-encoding mRNA without RNA amplification did not lead to detectable Ab staining of reporter protein. SFV3 mRNA transfection of DC followed by staining for bacterial β-gal demonstrated that >90% of DC expressed bacterial β-gal protein (Fig. 2). Controls without primary Ab or without β-gal-encoding mRNA gave no staining (Fig. 2 and data not shown). Primary DC transfection with DNA results in low numbers of cells making encoded protein (24–26), but we observed that relatively high levels of β-gal activity were produced with a pCMV-β-gal plasmid. DC were immunohistochemically stained for bacterial β-gal 24 h after plasmid transfection. Less than 1% of the cells stained with a mAb, demonstrating

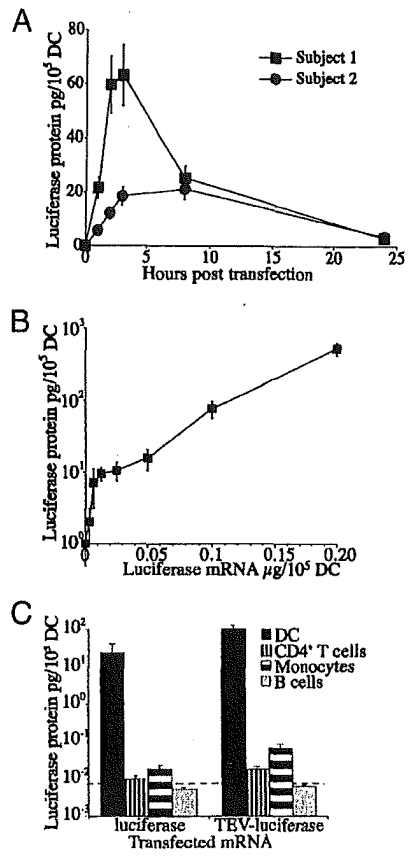

FIGURE 1. Luciferase-encoded mRNA transfection of DC results in transient, high level, and functional luciferase expression. Immature DC derived from cytokine-treated monocytes were transfected with lipofectin-complexed luciferase-encoding mRNA. Cells were transfected with 0.2 μg/50 μl mRNA and lysed at the given time points posttransfection (*A*) or cells were transfected with indicated amounts of mRNA and lysed at 4 h post transfection (*B*). DC, B cells, $CD4^+$ T cells, and monocytes were transfected with 0.2 μg/50 μl luciferase and TEV-luciferase mRNA and lysed 4 h later (*C*). Luciferase activity was quantitated by measuring light generation upon substrate cleavage. Luciferase protein concentration was calculated using a standard curve of purified luciferase protein. The dashed line in (*C*) represents the level of detection of the assay. DC were prepared in 10% FCS, 1% NHS, or AIM V serum-free medium, and all conditions gave similar results. Duplicate cultures and duplicate measurements per culture were performed. Error bars represent the SEM of the four values. Data are representative of at least four subjects for each DC culture condition.

that high levels of staining after mRNA transfection resulted from protein produced within the DC. These studies demonstrated that high levels of protein expression resulted from mRNA transfection of DC and that almost all of the cells took up and translated delivered mRNA.

Transfection of immature DC with gag mRNA leads to synthesis of cell-associated and -secreted gag protein

$CD4^+$ and $CD8^+$ T cell activation by a vaccine depends on Ag delivery to DC in a manner that loads both MHC class II and I molecules, respectively, with Ag-derived peptides. The loading of MHC class I molecules optimally occurs by processing intracellularly translated proteins, whereas MHC class II molecules obtain antigenic peptides through the processing of endocytosed proteins (reviewed in Refs. 51–53). Immature and mature DC and the 293T cell line were transfected with TEV-gag mRNA to analyze the

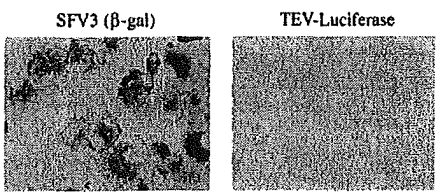

FIGURE 2. mRNA-transfected immature DC stain for encoded β-gal protein. DC were transfected with SFV3 mRNA or TEV-luciferase (control) mRNA. The cells were cytospun onto slides and immunohistochemically stained with bacterial β-gal-specific mAb 20 h after transfection. DC transfected with SFV3 and immunostained without primary mAb demonstrated no specific staining, similar to TEV-luciferase (data not shown). Data are representative of two separate experiments.

production and ratio of cell-associated and -secreted protein. The 5′ leader sequence of the tobacco etch viral genome was used as it promotes translation leading to higher levels of protein production from the same amount of mRNA (40). High levels of p24 Ag were found in both culture supernatant and cell lysate from transfected immature DC. The variability in gag protein produced by transfected immature DC was <8-fold across all subjects studied. Compared with mature DC and 293T cells, more p24 was cell associated than secreted in immature DC (Fig. 3). We hypothesized that this was due to uptake of released protein by endocytosis and macropinocytosis, processes present in immature DC and lost with DC maturation (36).

Gag mRNA-transfected DC induce a potent in vitro primary immune response

We next sought to determine whether delivery of Ag to DC by mRNA transfection could induce a primary immune response in vitro. Immature DC were pulsed with HIV gag mRNA or protein or infected with recombinant vaccinia virus-encoding gag and matured 6 h later with TNF-α and PGE_3. Mature DC were also pulsed with a series of overlapping 20-mer polypeptides corresponding to the entire p55 gag protein. DC were then cocultured with autologous T cells in the presence or absence of IL-2 and IL-7 for 2 wk. T cells were restimulated with DC pulsed in the same manner as the initial stimulation, except for vaccinia gag, which was restimulated with gag mRNA-loaded DC to distinguish vaccinia-specific responses, and the early activation markers, CD69 and IFN-γ,

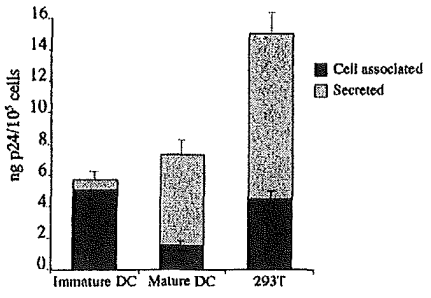

FIGURE 3. Expression of gag protein by cells transfected with gag-encoding mRNA. Immature and mature DC and 293T cells were transfected with gag-encoding mRNA (TEV-gag). Culture medium (secreted) (gray box) and cell lysate (cell-associated) (■) p24 protein was assayed by ELISA after 24 h. The mature DC produced more p24 protein than immature DC after transfection by TEV-gag mRNA. This was supported by comparing the kinetics of luciferase production after TEV-luciferase transfection of matched mature and immature DC. Triplicate cultures and duplicate measurements per culture were performed. Error bars represent the SEM of the six values. Data represent three separate experiments.

were analyzed on $CD4^+$ and $CD4^-$ T cells (as T cells were purified by negative selection from PBMC, the $CD4^-$ cells were assumed to be $CD8^+$ T cells). The percentage of $CD69^+$, IFN-γ^+ cells represented the frequency of Ag-specific cells generated during the primary immune response (54, 55). DC transfected with gag mRNA demonstrated an efficient induction of Ag-specific cells, typically 5–9% of $CD4^+$ or $CD8^+$ T cells. Unfortunately, a high level of background stimulation (1.7–4.2%) was observed. We thought this was likely due to the presentation of serum-derived Ags obtained during the DC culture period, which was performed in 1% NHS, which was excluded by purifying and culturing monocytes in serum-free medium (Aim V) to produce immature DC. These DC were phenotypically and functionally identical with DC produced in serum (56) (D. Weissman, unpublished observations). DC produced in serum-free medium were pulsed with mRNA, protein, vaccinia gag, or peptides and cultured with T cells as described above except that all steps, including T cell stimulation, were performed in serum-free medium. The $CD4^+$ and $CD8^+$ T cell primary immune responses initiated by gag mRNA-transfected DC were similar in magnitude to that generated in the presence of serum, whereas lower but still significant levels of background were observed (ranging from 0.2 to 2.2%) (Fig. 4). A low level of T cell activation above that observed for control DC-stimulated T cells was observed with gag protein or peptides or delivery of encoded gag by vaccinia virus (Fig. 4). Similar results were observed whether or not IL-2 and IL-7 were included during the primary stimulation. Thus, mRNA delivery of encoded gag to DC induced the activation and expansion of Ag-specific $CD4^+$ and $CD8^+$ T cells at a frequency generally observed after in vivo primary immune stimulation of mice (35).

mRNA pulsing induces DC maturation

DC, in their immature form, are efficient obtainers and processors of Ag, but are relatively inefficient at stimulating T cells. The ability to potently stimulate T cells occurs with maturation of DC.

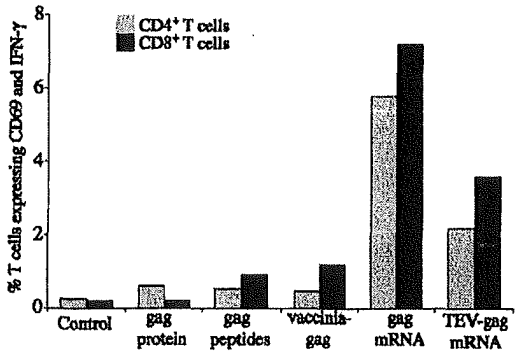

FIGURE 4. $CD4^+$ and $CD8^+$ T cell activation during an in vitro immune response was induced by gag-encoding mRNA-pulsed DC. Immature DC derived from PBMC of HIV-naive individuals were pulsed with gag mRNA, vaccinia-gag, or p55 gag protein, matured with TNF-α and PGE_3, and cocultured with autologous T cells for 2 wk. Mature DC were pulsed with peptides corresponding to the entire p55 gag protein before T cell coculture. T cells were restimulated with DC pulsed in the same manner as the first stimulation and analyzed for expression of cell surface CD4 and intracellular CD69 and IFN-γ by flow cytometry. DC generation and T cell stimulation were performed in serum-free medium without IL-2 and IL-7. As T cells were purified by negative selection, we assumed that the $CD4^-$ T cells were $CD8^+$ T cells. Data are representative of four experiments with and four without IL-2 and IL-7 in serum-free medium. Vaccinia encoding gag infection of DC was performed two times in serum-free medium without IL-2 and IL-7.

Human trials using Ag-loaded DC to induce T cell immunity, in addition to loading DC with protein or peptide, also delivered a maturation signal with monocyte-conditioned medium (14, 57). In addition to monocyte-conditioned medium, TNF-α, LPS, and CD40 ligand, dsRNA, have also been demonstrated to induce DC maturation (58, 59). We analyzed the effect of gag mRNA transfection on DC and observed efficient maturation as measured by expression of CD83 (a marker of mature DC; Ref. 60), increased CD80 and CD86 mean fluorescence (Fig. 5), and the loss of the ability to macropinocytose or endocytose (data not shown). The expression of DC maturation markers were not increased by pulsing DC with lipofectin alone (Fig. 5) or gag protein (data not shown).

TEV gag mRNA compared with gag mRNA produced four to five times more gag protein in transfected DC, often led to higher levels of expression of DC maturation markers, but, surprisingly, consistently produced lower levels of T cell stimulation (Fig. 4). Mature DC die as the next phase of their life cycle (2–4). An explanation for the discrepancy between TEV-gag and gag mRNA pulsing followed by maturation with TNF-α and PGE_3 of DC was that the decreased T cell stimulation was due to overmaturation of the DC with less efficient T cell stimulation. In fact, DC receiving both maturation signals had elevated levels of cell death measured by PI uptake compared with DC receiving only one maturation signal after 24 h. When DC were not matured with TNF-α and PGE_3 after TEV-gag mRNA transfection, thereby allowing the mRNA to deliver the maturation signal, higher levels (12–15%) of T cell activation were observed with almost no background activation (<0.25%) (Fig. 6). Levels of T cell activation above background, but much less than gag mRNA, were observed during a primary in vitro immune response when whole gag protein, gag peptides, or recombinant vaccinia-encoding gag were used to load DC with Ag.

mRNA-pulsed DC expand $CD4^+$ and $CD8^+$ memory T cells

Activation of memory cells results in the expansion of effector cells, capable of lysing targets or secreting cytokines, and memory cells, capable of homing to lymphoid organs, activating DC, and maintaining the pool of memory cells specific for an Ag (reviewed in Ref. 61). We measured the ability of DC to expand memory cells by restimulating T cells from primary immune response for 2 wk and measuring activation and IFN-γ expression after Ag-pulsed DC stimulation. A potent activation and expansion of gag-specific memory cells was observed during a second in vitro stimulation with gag mRNA-pulsed DC (Fig. 6).

mRNA-transfected DC induce potent CTL effectors in vitro

It has been demonstrated that the amount of antigenic peptide presented by a DC determines the type of $CD8^+$ T cell effector response, with low concentrations inducing CTL activity and higher concentrations leading to loss of CTL effector function and replacement with cytokine production (47). To determine the type of CD8 effector induced by delivering Ag through mRNA transfection of DC, CTL activity was analyzed after a primary in vitro immune response. Two weeks after primary stimulation, resting T cells were restimulated for 5 days with gag peptides, protein, or mRNA-pulsed DC to activate effectors. $CD8^+$ T cells were purified by negative selection and added to gag mRNA-loaded autologous DC targets (34). Efficient and specific lysis of gag-loaded autologous DC targets (Fig. 7) was observed after a primary in vitro immune response induced by gag or TEV-gag mRNA-pulsed DC. A comparison of target loading with gag mRNA vs peptides corresponding to the entire p55 gag protein demonstrated that mRNA loading produced superior targets, typically 2–3 times better ability to be lysed (data not shown), but also demonstrated that a pool of 20-mer overlapping polypeptides were efficient at loading target cells for lysis. Our more limited ability to induce a primary CTL response to peptide-loaded DC-stimulated T cells is likely due to the use of pooled peptides instead of selected peptides for each MHC haplotype as has been described (62, 63). Thus, gag mRNA-pulsed DC induced potent $CD8^+$ T cell effector activity that could be measured on autologous gag mRNA or gag peptide-pulsed DC targets.

Discussion

Delivering Ags in their DNA- or RNA-encoded forms represents a new approach to the induction of T cell immunity that depends

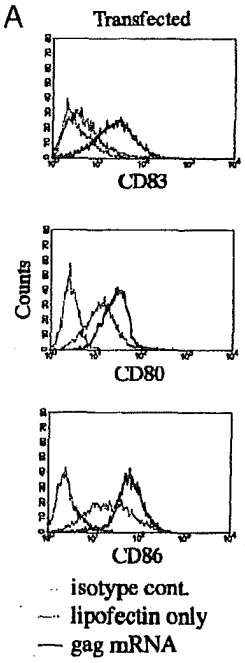

FIGURE 5. mRNA transfection of immature DC induces the maturation marker CD83 and up-regulates the costimulatory molecules CD80 and CD86. Immature DC were transfected with gag mRNA complexed with lipofectin or treated with lipofectin alone. Twenty-four hours later, cells were analyzed by flow cytometry (*A*). *B*, The percentage of positive cells and mean fluorescence are given for lipofectin alone, gag mRNA, and TNF-α plus PGE_3-treated DC. Data are representative of six separate experiments.

B

	CD83		CD80		CD86	
Treatment	% pos	mean Fl	% pos	mean Fl	% pos	mean Fl
lipofectin only	4.0	8.3	56.5	20.1	55.3	37.8
gag mRNA	42.8	32.3	89.6	31.4	94.1	76.2
TNF-α + PGE_3	61.6	85.2	82.4	28.6	81.9	56.4

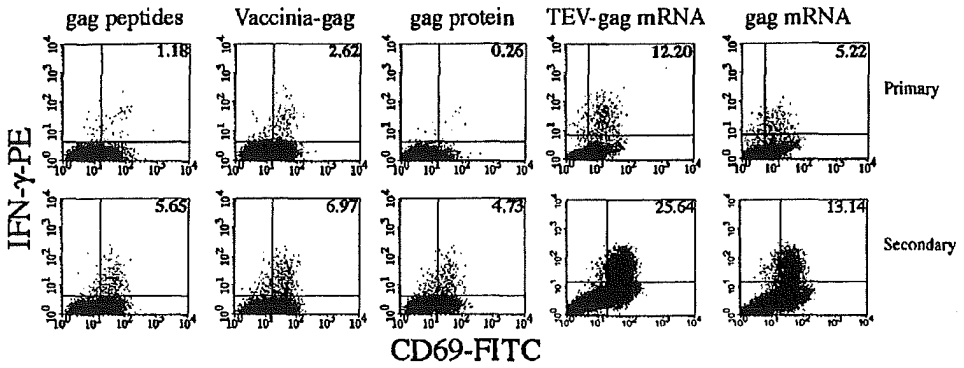

FIGURE 6. mRNA transfection of immature DC induces potent Ag-specific T cell activation in the absence of posttransfection DC maturation by TNF-α and PGE_2. Gag peptides and protein, vaccinia-gag, gag, and TEV-gag mRNA-pulsed DC were cocultured with autologous T cells from HIV-naive individuals for 2 wk, restimulated, and analyzed (primary immune response). Primary stimulated T cells were restimulated for 2 wk and reanalyzed (secondary immune response). Poly(A) homopolymer was used as a control. Cultures were performed in serum-free medium supplemented with IL-2 (40 U/ml) and IL-7. Histograms are gated on all T cells. Data presented were derived from two different subjects and matched for primary and secondary responses. Data are representative of three separate experiments.

upon the ability of the host to make antigenic proteins from delivered transgenes. In this report, we demonstrated that DC could be efficiently pulsed with mRNA encoding the HIV core protein gag, which then induced a potent primary in vitro T cell immune response. Most of the gag protein produced by immature DC, unlike other cell lines examined, remained within the DC after mRNA transfection. Intracellularly made protein is processed by the proteasome system into peptides that are loaded onto MHC class I molecules in the endoplasmic reticulum. Some of the gag protein was secreted and subsequently endocytosed by the DC, which is the pathway used to load MHC class II molecules. A significant activation and expansion of Ag-specific $CD4^+$ and $CD8^+$ T cells was observed, confirming loading of both MHC class I and II molecules by mRNA-encoding Ag transfection. Delivery of mRNA-encoding Ag to immature DC not only efficiently loaded both Ag presentation pathways, but also delivered a maturation signal to DC, converting them into potent T cell stimulators. The ability to deliver antigenic peptides for presentation to both $CD4^+$ and $CD8^+$ T cells and signal DC to mature and activate T cells has not been observed with other forms of Ag loading of DC.

The inefficiency of transfecting primary cells is multifactorial, including a restriction of plasmid reaching the nucleus and of transcribed mRNA from genes lacking introns being translated (64). The high efficiency of mRNA transfection compared with DNA transfection can be explained by the processes required to generate protein from each. With DNA transfection, the plasmid must enter the cell, be transported into the nucleus, become transcribed by cellular transcriptional machinery, and, finally, have the resulting mRNA transported into the cytoplasm. Additionally, the resulting mRNA must be in a form that can be translated (65). In a study of DNA transfection of HeLa cells, it was observed that 95% of the cells had plasmid in their cytoplasm, but only a proportion (30%) produced the encoded protein (66). If DNA and RNA entrance into a cell share a similar mechanism, it is likely that nearly all mRNA-transfected DC would have mRNA in their cytoplasm, and protein production would be dependent on the translational capacity of the cell. In fact, this is what we observed. mRNA-pulsed DC had encoded protein present in >90% of DC. Thus, mRNA transfection of DC results in cytoplasmic protein production in a majority of cells with subsequent loading of MHC class I molecules as measured by activation of CTL effector cells. At least in the setting of HIV gag, and likely with any protein that can be secreted or released, MHC class II molecule loading occurred, resulting in T helper cell activation likely through release and reuptake of protein into DC phagolysosomes.

The first step in the induction of T cell immunity by a vaccine is the delivery of Ag to DC. Immunity induced by DNA-encoding Ag immunization correlates with delivery of Ag to DC (10–13). Recently, two approaches to in vivo mRNA vaccination have been described. These approaches demonstrated new methods of delivering mRNA encoding a candidate Ag to nonlymphoid cells using an episomal fusion complex (67) and enhanced DC Ag uptake by inducing caspase-dependent apoptosis of nonlymphoid cells that had translated the mRNA (68). Another recent report demonstrated that complexed and naked mRNA injected into mice could induce CTL activity and specific Ab (69). The dichotomy presented by these studies is that the induction of primary immune responses in vitro, as described in this study and, recently, in another system (34), uses mRNA transfection of DC to produce protein within the DC and develop potent $CD8^+$ T cell immunity, whereas the in vivo studies above (67, 68) deliver mRNA in forms that enhance the uptake of encoded protein by DC. These approaches do not take advantage of the ability of intracellular protein from mRNA transfection of DC to enter the MHC class I Ag-processing pathway and, instead, rely on cross-priming for the activation of $CD8^+$

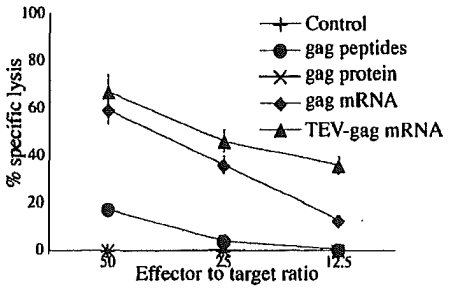

FIGURE 7. gag mRNA-loaded DC induce CTL effector activity after a single expansion in vitro. T cells, 2 wk after a primary stimulation, were restimulated with DC pulsed in a similar manner to the primary stimulation for 5 days. $CD8^+$ T cells were purified by negative selection and added to calcein- and Ag-loaded (gag mRNA transfection) DC targets for 3 h. Supernatants were analyzed by fluorometry for released calcein. Cultures were performed in triplicate and error bars indicate SEM. Data are representative of three experiments.

T cells. Alternatively, approaches that use in vitro isolation, growth, loading, and maturation of DC before delivery back to the patient preclude large scale use of in vitro DC-loading vaccines, suggesting that in vivo delivery of mRNA-encoding candidate Ags is needed. DC translated mRNA much more efficiently than other professional APC or primary cells (including vascular endothelial and smooth muscle cells; K. Karikó, unpublished observations) and almost all DC pulsed with mRNA made encoded protein. These findings, combined with the observations that DC induced a primary immune response in vitro after gag mRNA pulsing and mRNA transfection resulted in a signal that activated and matured DC, suggest that although injection of mRNA into a subject will result in widespread biodistribution, the Ag encoded by the mRNA will be preferentially produced in and presented by DC. This form of delivery of Ag to DC will direct encoded protein to the MHC class I processing pathway and, additionally, when a secreted protein is used or lysosomal signaling sequences are added (34), entrance into the MHC class II pathway will occur, inducing potent $CD4^+$ and $CD8^+$ T cell immune responses. The ability to deliver mRNA to DC in vivo as a vaccine approach is also attractive as it can be easily and inexpensively administered to large populations.

References

1. Pardigon, N., and D. Laouini. 1999. New millenium: the need for new vaccines. *C. R. Acad. Sci. Ser. III 322:913.*
2. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol. 9:271.*
3. Mehta-Damani, A., S. Markowicz, and E. G. Engleman. 1994. Generation of antigen-specific $CD8^+$ CTLs from naive precursors. *J. Immunol. 153:996.*
4. Caux, C., Y. J. Liu, and J. Banchereau. 1995. Recent advances in the study of dendritic cells and follicular dendritic cells. *Immunol. Today 16:2.*
5. Harrer, T., E. Harrer, S. A. Kalams, T. Elbeik, S. I. Staprans, M. B. Feinberg, Y. Cao, D. D. Ho, T. Yilma, A. M. Caliendo, et al. 1996. Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. *AIDS Res. Hum. Retroviruses 12:585.*
6. Rosenberg, E. S., J. M. Billingsley, A. M. Caliendo, S. L. Boswell, P. E. Sax, S. A. Kalams, and B. D. Walker. 1997. Vigorous HIV-1-specific $CD4^+$ T cell responses associated with control of viremia. *Science 278:1447.*
7. Connor, R. I., B. T. Korber, B. S. Graham, B. H. Hahn, D. D. Ho, B. D. Walker, A. U. Neumann, S. H. Vermund, J. Mestecky, S. Jackson, et al. 1998. Immunological and virological analyses of persons infected by human immunodeficiency virus type 1 while participating in trials of recombinant gp120 subunit vaccines. *J. Virol. 72:1552.*
8. Letvin, N. L. 1998. Progress in the development of an HIV-1 vaccine. *Science 280:1875.*
9. Girard, M., A. Habel, and C. Chanel. 1999. New prospects for the development of a vaccine against human immunodeficiency virus type 1: an overview. *C. R. Acad. Sci. Ser. III 322:959.*
10. Condon, C., S. C. Watkins, C. M. Celluzzi, K. Thompson, and L. D. Falo, Jr. 1996. DNA-based immunization by in vivo transfection of dendritic cells. *Nat. Med. 2:1122.*
11. Casares, S., K. Inaba, T. D. Brumeanu, R. M. Steinman, and C. A. Bona. 1997. Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. *J. Exp. Med. 186:1481.*
12. Porgador, A., K. R. Irvine, A. Iwasaki, B. H. Barber, N. P. Restifo, and R. N. Germain. 1998. Predominant role for directly transfected dendritic cells in antigen presentation to $CD8^+$ T cells after gene gun immunization. *J. Exp. Med. 188:1075.*
13. Chattergoon, M. A., T. M. Robinson, J. D. Boyer, and D. B. Weiner. 1998. Specific immune induction following DNA-based immunization through in vivo transfection and activation of macrophages/antigen-presenting cells. *J. Immunol. 160:5707.*
14. Kundu, S. K., E. Engleman, C. Benike, M. H. Shapero, M. Dupuis, W. C. A. Van Schooten, M. Eibl, and T. C. Merigan. 1998. A pilot clinical trial of HIV antigen-pulsed allogeneic and autologous dendritic cell therapy in HIV-infected patients. *AIDS Res. Hum. Retroviruses 14:551.*
15. Gilboa, E., S. K. Nair, and H. K. Lyerly. 1998. Immunotherapy of cancer with dendritic-cell-based vaccines. *Cancer Immunol. Immunother. 46:82.*
16. Minev, B. R., F. L. Chavez, and M. S. Mitchell. 1998. New trends in the development of cancer vaccines. *In Vivo 12:629.*
17. Ada, G. 1999. The coming of age of tumour immunotherapy. *Immunol. Cell Biol. 77:180.*
18. Tarte, K., and B. Klein. 1999. Dendritic cell-based vaccine: a promising approach for cancer immunotherapy. *Leukemia 13:653.*
19. Bronte, V., M. W. Carroll, T. J. Goletz, M. Wang, W. W. Overwijk, F. Marincola, S. A. Rosenberg, B. Moss, and N. P. Restifo. 1997. Antigen expression by dendritic cells correlates with the therapeutic effectiveness of a model recombinant poxvirus tumor vaccine. *Proc. Natl. Acad. Sci. USA 94:3183.*
20. Kim, C. J., T. Prevette, J. Cormier, W. Overwijk, M. Roden, N. P. Restifo, S. A. Rosenberg, and F. M. Marincola. 1997. Dendritic cells infected with poxviruses encoding MART-1/Melan A sensitize T lymphocytes in vitro. *J Immunother. 20:276.*
21. Szabolcs, P., H. F. Gallardo, D. H. Ciocon, M. Sadelain, and J. W. Young. 1997. Retrovirally transduced human dendritic cells express a normal phenotype and potent T-cell stimulatory capacity. *Blood 90:2160.*
22. Diao, J., J. A. Smythe, C. Smyth, P. B. Rowe, and I. E. Alexander. 1999. Human PBMC-derived dendritic cells transduced with an adenovirus vector induce cytotoxic T-lymphocyte responses against a vector-encoded antigen in vitro. *Gene Ther. 6:845.*
23. Ruppert, J., R. T. Kubo, J. Sidney, H. M. Grey, and A. Sette. 1994. Class I MHC-peptide interaction: structural and functional aspects. *Behring Inst. Mitt. :48.*
24. Van Tendeloo, V. F., H. W. Snoeck, F. Lardon, G. L. Vanham, G. Nijs, M. Lenjou, L. Hendriks, C. Van Broeckhoven, A. Moulijn, I. Rodrigus, et al. 1998. Nonviral transfection of distinct types of human dendritic cells: high-efficiency gene transfer by electroporation into hematopoietic progenitor- but not monocyte-derived dendritic cells. *Gene Ther. 5:700.*
25. Timares, L., A. Takashima, and S. A. Johnston. 1998. Quantitative analysis of the immunopotency of genetically transfected dendritic cells. *Proc. Natl. Acad. Sci. USA 95:13147.*
26. Akbari, O., N. Panjwani, S. Garcia, R. Tascon, D. Lowrie, and B. Stockinger. 1999. DNA vaccination: transfection and activation of dendritic cells as key events for immunity. *J. Exp. Med. 189:169.*
27. Chirmule, N., and S. Pahwa. 1996. Envelope glycoproteins of human immunodeficiency virus type 1: profound influences on immune functions. *Microbiol. Rev. 60:386.*
28. Reinhold, D., S. Wrenger, T. Kahne, and S. Ansorge. 1999. HIV-1 Tat: immunosuppression via TGF-β1 induction. *Immunol. Today 20:384.*
29. Subklewe, M., A. Chahroudi, A. Schmaljohn, M. G. Kurilla, N. Bhardwaj, and R. M. Steinman. 1999. Induction of Epstein-Barr virus-specific cytotoxic T-lymphocyte responses using dendritic cells pulsed with EBNA-3A peptides or UV-inactivated, recombinant EBNA-3A vaccinia virus. *Blood 94:1372.*
30. Ranieri, E., W. Herr, A. Gambotto, W. Olson, D. Rowe, P. D. Robbins, L. S. Kierstead, S. C. Watkins, L. Gesualdo, and W. J. Storkus. 1999. Dendritic cells transduced with an adenovirus vector encoding Epstein-Barr virus latent membrane protein 2B: a new modality for vaccination. *J. Virol. 73:10416.*
31. Conry, R. M., A. F. LoBuglio, M. Wright, L. Sumerel, M. J. Pike, F. Johanning, R. Benjamin, D. Lu, and D. T. Curiel. 1995. Characterization of a messenger RNA polynucleotide vaccine vector. *Cancer Res. 55:1397.*
32. Conry, R. M., A. F. LoBuglio, and D. T. Curiel. 1996. Polynucleotide-mediated immunization therapy of cancer. *Semin. Oncol. 23:135.*
33. Boczkowski, D., S. K. Nair, D. Snyder, and E. Gilboa. 1996. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. *J. Exp. Med. 184:465.*
34. Nair, S. K., D. Boczkowski, M. Morse, R. I. Cumming, H. K. Lyerly, and E. Gilboa. 1998. Induction of primary carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocytes in vitro using human dendritic cells transfected with RNA. *Nat. Biotechnol. 16:364.*
35. Mata, M., and Y. Paterson. 1999. Th1 T cell responses to HIV-1 Gag protein delivered by a *Listeria* monocytogenes vaccine are similar to those induced by endogenous listerial antigens. *J. Immunol. 163:1449.*
36. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med. 179:1109.*
37. Weissman, D., A. Rubbert, C. Combadiere, P. M. Murphy, and A. S. Fauci. 1997. Dendritic cells express and use multiple HIV coreceptors. *Adv. Exp. Med. Biol. 417:401.*
38. Erickson-Viitanen, S., J. Manfredi, P. Viitanen, D. E. Tribe, R. Tritch, C. A. D. Hutchison, D. D. Loeb, and R. Swanstrom. 1989. Cleavage of HIV-1 gag polyprotein synthesized in vitro: sequential cleavage by the viral protease. *AIDS Res. Hum. Retroviruses 5:577.*
39. Liljestrom, P., and H. Garoff. 1991. A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Biotechnology 9:1356.*
40. Gallie, D. R., R. L. Tanguay, and V. Leathers. 1995. The tobacco etch viral 5′ leader and poly(A) tail are functionally synergistic regulators of translation. *Gene 165:233.*
41. Ratner, L., A. Fisher, L. L. Jagodzinski, H. Mitsuya, R. S. Liou, R. C. Gallo, and F. Wong-Staal. 1987. Complete nucleotide sequences of functional clones of the AIDS virus. *AIDS Res. Hum. Retroviruses 3:57.*
42. Kariko, K., A. Kuo, E. S. Barnathan, and D. J. Langer. 1998. Phosphate-enhanced transfection of cationic lipid-complexed mRNA and plasmid DNA. *Biochim. Biophys. Acta 1369:320.*
43. Chakrabarti, S., J. R. Sisler, and B. Moss. 1997. Compact, synthetic, vaccinia virus early/late promoter for protein expression. *Biotechniques 23:1094.*
44. Earl, P. L., and B. Moss. 1991. *Current Protocols in Molecular Biology.* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds. Greene Publishing Associates & Wiley Interscience, New York, p. 16.7.1.
45. Steimer, K. S., J. P. Puma, M. D. Power, M. A. Powers, C. George-Nascimento, J. C. Stephans, J. A. Levy, R. Sanchez-Pescador, P. A. Luciw, P. J. Barr, et al. 1986. Differential antibody responses of individuals infected with AIDS-associated retroviruses surveyed using the viral core antigen p25gag expressed in bacteria. *Virology 150:283.*

46. Wong, C., M. Morse, and S. K. Nair. 1998. Induction of primary, human antigen-specific cytotoxic T lymphocytes in vitro using dendritic cells pulsed with peptides. *J. Immunother. 21:32.*
47. Valitutti, S., S. Muller, M. Dessing, and A. Lanzavecchia. 1996. Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. *J. Exp. Med. 183:1917.*
48. Viola, A., and A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science 273:104.*
49. Manickan, E., S. Kanangat, R. J. Rouse, Z. Yu, and B. T. Rouse. 1997. Enhancement of immune response to naked DNA vaccine by immunization with transfected dendritic cells. *J. Leukocyte Biol. 61:125.*
50. Souren, J. E., F. A. Wiegant, P. van Hof, J. M. van Aken, and R. van Wijk. 1999. The effect of temperature and protein synthesis on the renaturation of firefly luciferase in intact H9c2 cells. *Cell. Mol. Life Sci. 55:1473.*
51. Maffei, A., K. Papadopoulos, and P. E. Harris. 1997. MHC class I antigen processing pathways. *Hum. Immunol. 54:91.*
52. Castellino, F., G. Zhong, and R. N. Germain. 1997. Antigen presentation by MHC class II molecules: invariant chain function, protein trafficking, and the molecular basis of diverse determinant capture. *Hum. Immunol. 54:159.*
53. Djaballah, H. 1997. Antigen processing by proteasomes: insights into the molecular basis of crypticity. *Mol. Biol. Rep. 24:63.*
54. Maino, V. C., and L. J. Picker. 1998. Identification of functional subsets by flow cytometry: intracellular detection of cytokine expression. *Cytometry 34:207.*
55. Waldrop, S. L., C. J. Pitcher, D. M. Peterson, V. C. Maino, and L. J. Picker. 1997. Determination of antigen-specific memory/effector $CD4^+$ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. *J. Clin. Invest. 99:1739.*
56. Zhou, L. J., and T. F. Tedder. 1996. $CD14^+$ blood monocytes can differentiate into functionally mature $CD83^+$ dendritic cells. *Proc. Natl. Acad. Sci. USA 93: 2588.*
57. Dhodapkar, M. V., R. M. Steinman, M. Sapp, H. Desai, C. Fossella, J. Krasovsky, S. M. Donahoe, P. R. Dunbar, V. Cerundolo, D. F. Nixon, and N. Bhardwaj. 1999. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. *J. Clin. Invest. 104:173.*
58. Cella, M., M. Salio, Y. Sakakibara, H. Langen, I. Julkunen, and A. Lanzavecchia. 1999. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J. Exp. Med. 189:821.*
59. Verdijk, R. M., T. Mutis, B. Esendam, J. Kamp, C. J. Melief, A. Brand, and E. Goulmy. 1999. Polyriboinosinic polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells. *J. Immunol. 163:57.*
60. Zhou, L. J., R. Schwarting, H. M. Smith, and T. F. Tedder. 1992. A novel cell-surface molecule expressed by human interdigitating reticulum cells, Langerhans cells, and activated lymphocytes is a new member of the Ig superfamily. *J. Immunol. 149:735.*
61. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature 401:708.*
62. Wilson, C. C., W. C. Olson, T. Tuting, C. R. Rinaldo, M. T. Lotze, and W. J. Storkus. 1999. HIV-1-specific CTL responses primed in vitro by blood-derived dendritic cells and Th1-biasing cytokines. *J. Immunol. 162:3070.*
63. Zarling, A. L., J. G. Johnson, R. W. Hoffman, and D. R. Lee. 1999. Induction of primary human $CD8^+$ T lymphocyte responses in vitro using dendritic cells. *J. Immunol. 162:5197.*
64. Zabner, J., A. J. Fasbender, T. Moninger, K. A. Poellinger, and M. J. Welsh. 1995. Cellular and molecular barriers to gene transfer by a cationic lipid. *J. Biol. Chem. 270:18997.*
65. Matsumoto, K., K. M. Wassarman, and A. P. Wolffe. 1998. Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA. *EMBO J 17:2107.*
66. Tseng, W. C., F. R. Haselton, and T. D. Giorgio. 1997. Transfection by cationic liposomes using simultaneous single cell measurements of plasmid delivery and transgene expression. *J. Biol. Chem. 272:25641.*
67. Zhou, W. Z., D. S. Hoon, S. K. Huang, S. Fujii, K. Hashimoto, R. Morishita, and Y. Kaneda. 1999. RNA melanoma vaccine: induction of antitumor immunity by human glycoprotein 100 mRNA immunization. *Hum. Gene Ther. 10:2719.*
68. Ying, H., T. Z. Zaks, R. F. Wang, K. R. Irvine, U. S. Kammula, F. M. Marincola, W. W. Leitner, and N. P. Restifo. 1999. Cancer therapy using a self-replicating RNA vaccine. *Nat. Med. 5:823.*
69. Hoerr, I., R. Obst, H. G. Rammensee, and G. Jung. 2000. In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. *Eur. J. Immunol. 30:1.*